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(54) Title: USE OF HUMAN UMBILICAL CORD BLOOD FOR ADOPTIVE THERAPY

(57) Abstract

Human Umbilical Cord Blood (HUCB) cells have shown promising advantages over bone marrow (BM) cells for transplantation. This study indicates that in addition to their use for transplatation (i.e. engraftment), another potential clinical use for HUCB cells is as an adjuvant therapy to enhance both endogenous hematopoietic reconstitution and immunocompetence of the host.

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USE OF HUMAN UMBILICAL CORD BLOOD FOR ADOPTIVE THERAPY

DOMESTIC PRIORITY CLAIM

The priority is claimed of U.S. Provisional Application No. 60/067,459 filed on December 4, 1997, which is hereby incorporated by reference herein in its entirety.

GOVERNMENTAL SUPPORT

The present invention relates to the restoration of the hematopoietic and immune systems of a host, and more particularly to the practice of a method for achieving these objectives that does not involve engraftment of stimulatory agents or cells.

BACKGROUND OF THE INVENTION

The relatively slow progress in the area of gene therapy for life-threatening diseases such as leukemia and other genetic disorders makes bone marrow (BM) transplantation the treatment of choice. Unfortunately, BM transplantation is plagued with major clinical complications, especially graft-verses-host disease (GVHD). Measures to prevent GVHD in BM transplant patients have been attempted by depletion of particular BM cell populations (e.g. T cells), and also by transplantation of only BM stem cells. There are, however, disadvantages with these strategies, such as the requirement for considerably large amounts of donor BM cells, graft failure and reocurrence of malignancy [1,2]. The clinical complications that are associated with BM transplantation imply a requirement for alternative strategies. Human Umbilical Cord Blood (HUCB) is a rich source of hematopoietic stem cells and, compared to BM, it has more repopulation capabilities [3,4]. In fact, HUCB cells are now being widely used for transplantation in a variety of diseases, including patients undergoing therapy for hematologic disorders [5-7].

Successful engraftment of HLA mismatched HUCB cells has been reported, accompanied by little or no symptoms attributable to GVHD [8]. Perhaps, this lack of a requirement for an exact donor-recipient HLA match may be partly responsible for the successful use of HUCB in BM transplantation [9]. This may be partly explained by the immunologic immaturity and reduced functional properties of T-cells and other immune cells present in HUCB [10-14]. Another major advantage for use of HUCB cells is their low incidence of infection by cytomegalovirus and Epstein-Barr virus, both of which are associated with severe complications in BM transplantation [15,16]. However, despite the advantages of HUCB over BM cells for transplantation, there is some evidence which indicates that HUCB cells engraft slower than BM cells [17].

SUMMARY OF THE INVENTION

In accordance with the present invention, a method is disclosed for promoting the growth of the hematopoietic cells of a host comprising administering to the host an effective amount of human umbilical cord blood cells, active fragments thereof, mimics thereof and agonists thereof. The method of the invention contemplates the circumstance where said host has previously undergone therapeutic treatment that has caused myelosuppression, so that the method comprises a method for restoring the host hematopoietic system.

In a particular embodiment of the invention, the human umbilical cord blood cells, active fragments thereof, mimics thereof and agonists thereof, may be administered to the host by injection. One of the advantages of the invention is that this route of administration does not result in adverse sequelae such as the development of graft versus host disease. Rather the human umbilical cord blood cells promote the stimulation of the host progenitor cells to reconstitute their numbers.

A further aspect of the invention comprises the administration of human umbilical cord blood cells as a form of adjuvant therapy to restore the immune system of a host that may have been suppressed as by exposure to chemotherapy *e.g.* incident to cancer treatment. Again, the adverse results of cell transplant therapy may be avoided while achieving the salutary results of restoration of the normal immune response of the host.

A still further aspect of the invention contemplates a course of therapy wherein said human umbilical cord blood cells, active fragments thereof, mimics thereof and agonists thereof are co-cultured *ex vivo* with a quantity of the hematopoietic cells of said host, and the hematopoietic cells so treated are thereafter reintroduced into the host. For example bone marrow cells of the host may be withdrawn from the host and co-cultured with HUCB cells and then re-introduced to the host. Even

this therapy would avoid the introduction of cells from a donor and the possibilities for rejection or other complication that attend such transplantation.

Accordingly, the present invention extends to the use of human umbilical cord blood cells as a part of an adjuvant therapy given with HLA-matched bone marrow for patients who require such transplantation. A further application would be to administer the human umbilical cord blood cells as an adjuvant therapy to patients who receive high dose chemotherapy or radiotherapy and either autologous or allogeneic bone marrow cells.

A yet further aspect of the invention would be to apply the administration of human umbilical cord blood cells to treat patients who suffer from hematopoietic and/or immunologic deficiencies resulting from genetic abnormalities, as well as those conditions that may arise from disease-related or trauma-related deficiencies, such as, by way of non-limiting example, the following: trauma - to stimulate endogenous hematopoiesis; radiation injury - *e.g.* nuclear accidents; infectious diseases which cause myelosuppression and/or immunosuppression; innate (genetic) or acquired immunodeficiency diseases; innate or acquired (drug-induced) anemias; induction of tolerance for solid organ transplants; and non-malignant diseases associated with aging, *e.g.* osteoporosis.

Accordingly, it is a principal object of the invention to provide a method for treating deficiencies in hematopoietic and immune condition that is efficient and efficacious.

It is a further object of the invention to provide a method as aforesaid that reduces the likelihood of host rejection.

Other objects and advantages will become apparent from a review of the ensuing description which proceeds with reference to the following illustrative drawings.

DESCRIPTION OF THE DRAWINGS

Figure 1. WBC counts in peripheral blood and CFU-GM in the BM and spleen of HUCB-injected mice. NK cell-depleted SJL/J mice were lethally irradiated and then injected with either HUCB cells or vehicle. At various times, either peripheral blood WBC counts were determined (**A**) or mice were sacrificed and the number of CFU-GM in the BM and spleen was determined in clonogenic assays (**B**) as described in Materials and Methods. The number of CFU-GM colonies in the BM and spleen of normal, age-matched mice was 68 ± 4 and 30 ± 6 respectively; $n=9$, $\pm$SE.

Figure 2. Erythropoietic indices in peripheral blood of HUCB-injected mice. NK cell-depleted SJL/J mice were lethally irradiated and then injected with either HUCB cells or vehicle. At various times, a sample of peripheral blood was obtained for determination of RBC counts, and Hgb and Hct levels.

Figure 3. CFU-GM and WBC in peripheral blood in HUCB-injected mice that received 8.0 Gy irradiation. NK cell-depleted SJL/J mice were irradiated with 8.0 Gy and then injected with either HUCB cells or vehicle. At various time periods mice were either **A**) bled for WBC counts or, **B**) sacrificed for CFU-GM determination in the spleen and BM.

Figure 4. Allogeneic responses by lymphoid cells from HUCB-injected lethally irradiated SJL/J mice. NK-depleted SJL/J mice ($H\text{-}2^s$) were lethally irradiated and then injected with either HUCB mononuclear cells or syngeneic BM. At day 17 after injection, lymph node cells from

these mice were stimulated with γ-irradiated allogeneic spleen cells obtained from either Balb/c ($H\text{-}2^d$) or Balb.B ($H\text{-}2^b$) mice. The responses of lymph node cells obtained from age-matched, unirradiated control mice are shown for comparison. Cell proliferation, shown on the y-axis as cpm $x10^3$, was based on the amount of [^{3}H]TdR incorporated during the last 16-18 h of a 96 h incubation. Details of the procedure are described in the Materials and Methods.

Figure 5. Proliferative responses to T- and B- cell mitogens by splenocytes from HUCB-injected mice that were give a lower dose radiation. NK cell-depleted SJL/J mice were irradiated with 8.0 Gy and then injected with either HUCB cells or vehicle. At 3 and 9 wks after injection, splenocytes were cultured with either Con A or LPS for 72 h. During the final 16-18 h, cell proliferation was determined by [^{3}H]TdR incorporation. The Δ cpm are represented on the y-axis. Details of the procedure are described in the Materials and Methods.

Figure 6. Proliferative responses to Mouse Mammary Tumor Virus superantigen (MMTV) by lymphoid cells from HUCB-injected SJL/J mice that were given a lower dose radiation. Lymph node cells were obtained from mice that were irradiated with 8.0 Gy and then injected with either HUCB cells or vehicle. Cells were cultured with vSAg-expressing γ-irradiated RCS tumor cells for 96 h. During the final 16-18 h, cell proliferation was determined by [^{3}H]TdR incorporation. The Δ cpm are represented on the y-axis. Details of the procedure are described in the Materials and Methods.

Figure 7. Effect of HUCB cells on 5-FU treated BM mononuclear cells in LTC-IC assays.

Human BM stromal cells were irradiated (150 Gy) one day prior to co-culture with 5-FU treated BM mononuclear cells with or without γ-irradiated (100 Gy) HUCB mononuclear cells (10, 8, 5, 2, 1/well). At various time periods, cells from each well were trypsinized and analyzed for CFU-GM in short term clonogenic assays. The change (Δ) in CFU-GM is represented at each time point as the mean (±SD) of four different experiments. In each of the four experiments, cultures were performed in duplicate. At each time period, the number of colonies obtained for cultures at each of the 5 cell densities was totalled and the numbers of CFU-GM/26 5FU- cells are represented on the y-axis. CFU-GM were not detected in cultures containing only γ-irradiated HUCB mononuclear cells. Details of the procedure are described in the Materials and Methods.

DETAILED DESCRIPTION OF THE INVENTION

A compelling problem that continues to frustrate clinicians is the untoward side effects that accompany conventional forms of treatment which result in myelosuppression in their patients. Since many therapeutic agents target cells of the hematopoietic system, efforts to identify ways to restore the hematopoietic cells which are damaged in patients that receive these treatments are of paramount importance. We are exploring the potential use of Human Umbilical Cord Blood (HUCB) cells for adoptive therapy in such patients. There is increasing clinical use of HUCB cells to treat patients suffering from many diseases for which restoration of the hematopoietic system is required. In most hospitals, HUCB is still considered a medical waste product and is routinely discarded. However, the use of HUCB cells has numerous medical, practical and economic advantages over bone marrow for these purposes.

In most of the instances where HUCB cells have been transplanted to patients, great care is usually taken to select a donor with a close histocompatibility match to the recipient to ensure engraftment of the transplanted cells. However, our preliminary studies, using an experimental mouse model, suggest that in addition to engraftment, transplanted HUCB cells may also facilitate endogenous hematopoiesis by the recipient's own progenitor cells. In this model, mice are rescued from the lethal effects of high-dose irradiation by injection of HUCB. Within several weeks after injection of HUCB cells, these mice are not only reconstituted hematopoietically, but they are fully immunocompetent. Without such therapy, irradiated mice succumb due to hematopoietic or immunologic failure. Since there is little evidence for the presence of either human cells or even human DNA in these mice, long-term survival of these animals is not due to engraftment of HUCB cells. Rather, injection of HUCB cells into these lethally irradiated mice leads to stimulation of the recipients own surviving progenitor cells to begin the process of hematopoietic reconstitution. We have also established an in vitro model (modified LTC-IC assay) using human 5-Fluorouracil (5-FU) treated bone marrow cells co-cultured with irradiated HUCB and, despite allogeneic differences, HUCB cells are still able to promote accelerated colony formation (CFU-GM) from the 5-FU treated marrow progenitor cells. Although others are using HUCB in lieu of BM with the hope of engraftment, this new mechanism to stimulate endogenous hematopoiesis has, heretofore, not been described.

Thus, our preliminary results suggest the exciting possibility that even despite histocompatibility mismatches, HUCB cells (or their products) can be used as an adjuvant therapy in patients to help replenish the hematopoietic and immunologic progenitor cells that are damaged as a result of conventional forms of treatment, such as radiation or chemotherapy. We are now beginning to define the mechanisms by which HUCB cells mediate their hematopoiesis-enhancing effects, and we believe the results of our investigation will help to establish a new form of adjuvant therapy that is beneficial to many patients in need of hematopoietic/immunologic reconstitution due to various disease-related or treatment-induced deficiencies.

We previously reported that HUCB cells can increase the survival of lethally irradiated SJL/J mice compared to non-injected mice [18,19]. Long-term engraftment of the HUCB cells did not appear to be responsible for survival, suggesting that other mechanisms were operative. We therefore investigated possible mechanisms by which HUCB cells might enhance endogenous hematopoietic reconstitution. In the present study, we took advantage of the fact that HUCB cells do not permanently engraft in the SJL/J mice, and used this model to determine other functions for HUCB cells. We specifically examined whether HUCB cells can enhance endogenous hematopoietic reconstitution by residual, radioresistant host BM cells in irradiated SJL/J mice. We have also determined whether HUCB cells can function as an immune adjuvant. This was addressed by studying the responses of lymphoid cells obtained from HUCB-injected mice to: 1) T-cell and B-cell polyclonal activators, 2) alloantigens and, 3) a syngeneic B-cell lymphoma that stimulates through a mouse mammary tumor viral-encoded superantigen, Mtv-29 (vSAg) [20]. A clinical relevance for the results obtained in the mouse model has also been addressed in this study using a stem cell assay.

Materials and Methods

Cytokines and antibodies

Recombinant murine granulocyte-macrophage colony stimulating factor (rMuGM-CSF) was kindly provided by the Immunology Department of Genetics Institute (Cambridge, MA). Murine monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (IgG1),

murine monoclonal phycoerythrin (PE)-conjugated anti-human CD38 (IgG1) and, PE- and FITC-conjugated isotype controls were purchased from Caltag Laboratories (Burlingame, CA). Anti-CD45, CD3 and CD10 were conjugated to FITC and anti-CD19, CD14 and CD56 were conjugated to PE. All were obtained from Becton Dickinson Immuncytometry Systems (San Jose, CA).

Human Umbilical Cord Blood

Approximately 15-50 ml of HUCB was collected into citrate phosphate dextrose (Sigma, St Louis, MO). Deliveries were routine, and subjects had no underlying disease or infection. The collection and use of HUCB for this study was reviewed and approved by the Institutional Review Board of UMDNJ-New Jersey Medical School, Newark, NJ. Mononuclear cells were separated by Ficoll Hypaque (Sigma) density gradient centrifugation within 24 h of collection.

Adoptive Transfer

Female SJL/J mice, 6-8 wks, were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed in the AAALAC-accredited Research Animal Facility at UMDNJ-New Jersey Medical School, Newark, NJ. Mice were depleted of natural killer (NK) cells by retro-orbital injection (i.v.) of 100 μl rabbit anti-asialo GM1 (Wako Pure Chemicals, Osaka, Japan). Two weeks beyond this injection, NK cells remain undetectable, based on a cytotoxicity assay that utilizes splenic effector cells and the NK-susceptible target cells, YAC-1 [18,21].

Twenty four hours later, mice were irradiated either lethally (9.5 Gy) or sublethally (8.0 Gy) by a cesium source (Mark 1 model 68-A-3 gamma irradiator, J.L. Shepherd, San Fernando,

CA). After 1-2 h, mice were injected i.v. with 10^7 HUCB mononuclear cells resuspended in PBS. Control mice were injected with comparable volume of PBS (vehicle control). Mice were then housed in a laminar flow environment in sterile cages with sterile bedding, food and water. At various times thereafter, mice were analyzed for routine peripheral blood indices, lymphocyte functional assays, and granulocyte-macrophage colony-forming units (CFU-GM) in BM and spleen.

Immunofluorescence assays

The phenotypic profile for the expression of CD34, CD38, CD45, CD3, CD19, CD14, CD10 and CD56 in HUCB mononuclear cells was determined in random samples. Cells were labeled for 30 mins at 4°C with specific fluorescein (FITC)- or phycoerythrin (PE)-conjugated antibodies. After labeling, cells were washed to remove unbound antibodies, fixed by resuspending in 1% paraformaldehyde, and then analyzed by FACScan.

Clonogenic assays

Single cell suspensions from mice were prepared from either the femurs or spleens and then used in clonogenic assays for CFU-GM as described [22]. Briefly, cells were resuspended in culture medium and then plated in duplicate in methylcellulose at 10^5/plate in a total volume of 1 ml. Due to the low numbers of cells recovered in non-injected, lethally irradiated mice, for these cultures, the total numbers of recovered cells from two femurs (up to $3x10^5$) were plated in a single dish. Cultures were supplemented with 4 U of rMuGM-CSF. Colonies $\geq$20 cells were enumerated at day 10 of culture.

Lymphocyte Responses

A single cell suspension of responder cells (R) was prepared from the lymph nodes or spleen of the HUCB-injected or non-injected SJL/J mice. Responder cells were cultured with stimulators (S) obtained from either syngeneic γ-irradiated (7.5 Gy) B-lymphoma cells expressing vSAg (R/S=4), γ-irradiated (2.5 Gy) allogeneic ($H2^b$) spleen cells (R/S = 1) or the following mitogens: concanavalin A (Con A) at 1 μg/ml; lipopolysaccharide (LPS) at 5 μg/ml. Both mitogens were purchased from Sigma. Cultures stimulated with lymphoma cells were incubated for a total of 96 h and those stimulated with either allogeneic cells or mitogens were incubated for a total of 72 h. Cell proliferation was based on the incorporation of 1 μCi tritiated thymidine (specific activity 1.9 Ci/mM; Amersham Life Inc., Arlington Height, IL). ^{3}H-TdR incorporation was determined by harvesting cells onto glass fiber filters with an automated harvester. For each experiment, stimulation was performed in triplicate. The background cpm (responder cells alone or responder+stimulator cells alone) was subtracted from the cpm of stimulated cultures (Δ cpm).

BM stroma

BM aspirate was obtained from the posterior iliac crest of normal healthy volunteers. Samples were immediately placed into Iscove's medium (Life Technologies, Grand Island, NY) containing 50 U/ml preservative-free heparin. Informed consent was obtained from each donor according to the guidelines of the Institutional Review Board of UMDNJ-New Jersey Medical School, Newark, NJ.

BM aspirate cells ($4x10^6$) were cultured in 12-well plates (Corning Costar, Cambridge,

MA) in a total volume of 2 ml medium which consisted alpha minimal essential media (α-MEM) (Life Technologies) containing 12.5% FCS (Hyclone Laboratories, Logan, UT), 12.5% horse serum (Hyclone Laboratories), 10^{-7}M hydrocortisone (Sigma), 10^{-4}M 2-ME (Sigma) and 1.6 mM glutamine (Cellgro, Mediatech) (stromal medium). Cultures were incubated for 3 days at 33°C after which the mononuclear cells (BMNC) were separated from the non-adherent population by Ficoll-Hypaque density gradient centrifugation. BMNC were replated into culture flasks, which were reincubated with weekly 50% change of medium until confluency occurred.

Long-term culture-initiating cell assay (LTC-IC)

Confluent BM stroma prepared in 12-well plates were irradiated with 150 Gy that was delivered by a cesium source (Mark 1 Model 68-A-3). After 24 h, non-adherent cells were replaced with fresh media containing quiescent BM mononuclear cells (1-10/well). Parallel cultures consisted of wells with 10^5 γ-irradiated (100 Gy) HUCB cells. The radiation dose was established in LTC-IC assays with HUCB cells that were subjected to various dose of radiation (30-150 Gy). HUCB cells that were given less than 100 Gy proliferated in culture. During the culture period, 50% stromal medium was replaced weekly. At various time periods, cells from each well were trypsinized and cultured in duplicate in short term BM cultures.

Quiescent BM mononuclear cells were prepared by incubating cells with 5-Fluorouracil (5-FU) (Hoffman La Roche Inc., Nutley, NJ). This drug preferentially kill cells in cycling phase, while the quiescent population remains viable [23]. Cells (10^7) were resuspended in 5 ml α-MEM containing 20% FCS and 200 μg/ml 5-FU for 7-10 days. The cycling states of the cells were determined by pulsing 10^5 cells with 1 μCi [^{3}H]TdR (35 Ci/mM, ICN Biomedicals Inc.,

Irvine, CA) for 24 h. [^{3}H]TdR incorporation was determined as described for lymphocyte responses. By day 7, the dpm plateau at 215±24.

Results

We have previously shown [18,24] that a majority of lethally irradiated, HUCB-injected mice survived for a significant period beyond the time when non-HUCB-injected mice succumbed. Indeed, 40% of HUCB-injected mice survived until 180 days, compared to 0% survival in irradiated non-HUCB-injected mice. Since evidence of permanent engraftment of HUCB cells was not obtained in these mice, it appeared that endogenous hematopoietic repopulation was responsible for the long-term survival. We therefore addressed the mechanism by which such endogenous reconstitution might occur in HUCB-injected mice.

Phenotypic and Localization properties of injected HUCB cells

The phenotypic profile within the samples of HUCB mononuclear cells used in the study were determined in seven randomly selected samples. We measured the distribution of progenitor/stem cells (CD34), more matured progenitors (CD34/CD38), T-cell (CD3), B-cell (CD19), Thy1 (CD45) and NK cells (CD56). The phenotypic distribution within the mononuclear fractions used in our studies (Table 1) was consistent with published reports [25].

NK cells can affect hematopoietic activity in cord blood cells [26]. However, in this study, we did not attempt to engraft HUCB cells in the mice. Therefore, the significance of the 4% NK cells within the HUCB cells was not a concern for this particular model. Furthermore, the human NK cells would be irrelevant to the long-term endogenous hematopoietic

reconstitution in the mice, since the repopulating cells are not of human origin [18,24]. However, we previously observed that recipient murine NK cells can affect endogenous hematopoietic reconstitution in HUCB-injected mice [24]. Therefore, recipient mice were depleted of NK cells prior to transfer of HUCB cells.

We next determined the initial anatomic localization of the HUCB cells following i.v. injection. ^{51}Cr-labeled HUCB cells were injected into mice that had received 9.5 Gy and on days 1 and 2, groups of animals were sacrificed, and the radioactivity in various tissues was determined as a percentage of the injected cpm (Table 2). Control irradiated mice received syngeneic ^{51}Cr-labeled BM cells. Following i.v. injection of HUCB cells, the highest percentage of injected cpm (50%) was found in the liver on day 1, and this percentage was essentially unchanged on day 2. When syngeneic BM cells were injected, high counts (15%) were also found in the liver, but by day 2, this dropped to about 9% of injected cpm. Differences between HUCB-injected and syngeneic BM-injected recipients were also noted on day 1 for spleen (2.5% vs 7.2%), BM (0.14% vs 0.5%), and lung (0.07% vs 0.7%). However, by day 2, the differences between these groups in these organs decreased.

Effects on hematopoietic activity by HUCB in lethally-irradiated mice

Due to the radioresistance of NK cells, and their influence on hematopoiesis [27,28], mice were injected with an anti-NK antibody 24 h prior to administration of lethal radiation (9.5 Gy). This was followed by injection with either HUCB mononuclear cells or vehicle. At various time periods up to 3 weeks, peripheral blood indices were determined at selected intervals. In both HUCB-injected and vehicle-injected mice, there was a precipitous drop in the WBC count

to a nadir on day 10 (Figure 1A). After day 10, however, HUCB-injected mice showed accelerated return of WBC in comparison to non-injected mice. Indeed, by days 15-18, the peripheral blood WBC counts were 2200/μl in HUCB-injected mice, compared to only 500/μl in non-injected mice (Figure 1A). The WBC counts in normal age-matched controls were 8.8$\pm$0.6/μl (n=26, $\pm$SE). In contrast (Figure 2), erythropoietic related peripheral blood cell parameters exhibited little, if any decreases at day 5 in comparison to the levels observed in age-matched controls (n=26, $\pm$SE; Hct: 32$\pm$1%; Hgb: 11.6$\pm$0.4g; RBC: 7$\pm$0.2x10^6/μl). There was a slight decrease in these parameters in both groups of mice on day 10. Beyond day 10, however, whereas the red cells indices continued to fall in the non-HUCB-injected mice, these values increased to normal levels in mice that received HUCB cells.

We next studied myelopoiesis in the spleen and BM from both groups of mice (HUCB-injected and non-injected) using clonogenic cultures that contained 10^5 cells/ml. Based on the localization patterns, we cultured cells that were obtained from the liver, spleen, and BM. In BM and spleen, there was an increase in CFU-GM at day 10 that continued through days 15-18 (Figure 1B). By this time, the numbers of CFU/10^5 cells (60 and 40 in BM and spleen, respectively), were approaching the levels measured in tissues from normal age-matched control SJL/J mice (68$\pm$4 for BM and 30$\pm$6 for spleen, $\pm$SE; n=9). At none of these time intervals, however, was CFU-GM detected in cells taken from the livers of these mice. CFU-GM were also undetectable at these times in BM or spleens of vehicle-injected mice, despite the plating of up to 3x10^5 cells per culture.

We ruled out the possibility that the injected HUCB cells might contribute to the number of CFU-GM measured in the clonogenic assays. HUCB cells were used for CFU-GM assays in

which rMuGM-CSF was added. No colony growth occurred when HUCB cells were included with rMuGM-CSF, but they responded well to rhuGM-CSF in these assays (data not shown). Therefore, it is unlikely that the injected HUCB are a direct source of any of the CFU-GM assayed with the cells obtained from the mice in these experiments. Overall, these results indicate that HUCB cells are clearly involved in the process of hematopoietic recovery observed in the γ-irradiated mice, and appear to enhance the ability of surviving murine stem cells to begin endogenous repopulation.

Hematopoietic activity in lower dose γ-irradiated mice

We next determined if the period in which HUCB potentiates hematopoietic recovery could be shortened in mice that received a lower dose of radiation. Our rationale here was that at a lower dose, the surviving murine hematopoietic cells would include totipotent and multipotent cells compared to only totipotent cells remaining in lethally irradiated mice. Thus, following HUCB cell injection, the recovery period should be shorter. A secondary reason for using a lower dose was to be able to analyze the non-injected controls for a longer period of time, since at 9.5 Gy, practically all of the vehicle-injected mice succumbed between 2 and 3 weeks after irradiation. For these experiments, NK-depleted mice were irradiated with 8.0 Gy and then injected i.v. with 10^7 HUCB cells. At various time periods, WBC levels and CFU-GM in BM and spleen were determined. Although WBC levels and CFU-GM in HUCB-injected mice were greater than in vehicle-injected animals, the differences were not statistically significant ($p > 0.5$) (Figures 3A and 3B). These results indicate that with a lower dose of radiation, the level of endogenous reconstitution determined by the levels of progenitors and differentiated cells

are comparable for mice that were injected with either HUCB cells or vehicle.

Recovery of lymphocyte function in lethally irradiated, HUCB-injected mice

Prompted by the accelerated recovery of WBC and CFU-GM seen in lethally irradiated mice injected with HUCB cells, we also determined the recovery of lymphocyte function as measured by the ability of spleen cells to mount *in vitro* proliferative responses to murine alloantigens.

Three weeks after lethal irradiation and injection with either syngeneic BM or HUCB cells, SJL ($H\text{-}2^s$) splenic responder cells were stimulated in mixed lymphocyte reaction (MLR) with irradiated Balb.B ($H\text{-}2^b$) or Balb/c ($H\text{-}2^d$) stimulator cells. As shown in Figure 4, spleen cells from HUCB-injected mice proliferated in response to alloantigens to a similar degree as spleen cells taken from syngeneic BM-injected mice. None of the mice that received 9.5 Gy irradiation alone survived for 3 weeks in this series of experiments. These results demonstrate that injection of HUCB cells into lethally irradiated mice also influences lymphopoiesis, causing a recovery of mature, alloantigen-responsive lymphocytes within a time period similar to that observed for erythroid and myeloid compartments (Figures 1A, 1B and 2).

Recovery of lymphocyte function in mice that were given a lower dose radiation

In mice irradiated with 8.0 Gy, since no significant differences were observed in the myelopoietic compartment between the HUCB-injected mice irradiated with the lower dose and the non-injected controls (Figures 3A and 3B), we determined if HUCB cells can influence their immunocompetence levels. The data presented in Figure 5 indicate that at 3 weeks after

administration of 8.0 Gy, responses to the polyclonal lymphocyte activators Con A (T-cell) and LPS (B-cell) are more prominent in irradiated mice that received HUCB cells than in mice that were irradiated, but not injected with HUCB cells. At 9 weeks after irradiation, T-cell responses to Con A were comparable in both groups of mice, but B-cell responses to LPS in the HUCB-injected mice were closer to the response of age-matched unirradiated controls than mice that only received irradiation (Figure 5).

In Figure 6, the proliferative response of cells from these same mice to a syngeneic B-cell lymphoma was measured. Tsiagbe, et al [29] have shown that the response of SJL lymphoid cells to these lymphoma cells is stimulated by the expression of a mammary tumor provirus (Mtv)-encoded superantigen (vSAg) on the tumor cells (Mtv-29). Furthermore, the Mtv-29 vSAg stimulates T-helper (TH) cells that use a specific ß-chain (Vß16) in their T-cell receptor [30]. The data in Figure 6 show that the presence of tumor-responsive Vß16+ TH cells is comparably low in both groups of mice at 3 weeks after radiation. At 9 weeks, however, the Vß16+ TH cell response to syngeneic lymphoma cells is significantly reconstituted only in the irradiated mice that received HUCB cells, although the response was not the same as that of age-matched normal control mice.

Overall, these results suggest that the ability to mount polyclonal T- and B- cell responses is reconstituted earlier in 8.0 Gy irradiated mice that receive HUCB cells. This is in keeping with the results seen at this dose with the recovery of cells of other hematopoietic lineage (i.e. erythroid and myeloid). However, the results using more specific stimuli, such as the ability to mount a clonally restricted TH cell response to Mtv-vSAg, suggest that the return of selected, antigen-specific lymphocyte subsets proceeds more rapidly in irradiated mice that also receive

HUCB cells. To what degree this is true for other clonal populations of antigen-specific lymphocytes remains to be fully determined.

Effects of irradiated HUCB cells in LTC-IC cultures

Although the rescue of lethally irradiated mice by injection of HUCB cells demonstrated the biological relevance of the model, we could not exclude the possibility that some of the observed hematopoietic-inducing results were due to a "xenogeneic" effect. Therefore, we next determined if the ability of HUCB cells to stimulate hematopoiesis in the xenogeneic murine model could also be demonstrated with allogeneic human hematopoietic stem cells, using the LTC-IC assay.

Modified LTC-IC assays were performed with quiescent human BM cells in the presence or absence of γ-HUCB mononuclear cells. Beginning on day 10, cells from each well were trypsinized and the number of CFU-GM was determined in short term clonogenic assays. As shown in Figure 7, the presence of γ-HUCB cells considerably shortened the period by which the quiescent (5-FU treated) human stem cells proliferated. CFU-GM were detected by day 15 in γ-HUCB-containing cultures and maximal CFU-GM were observed at day 30. In contrast, in control cultures without HUCB, CFU-GM were not detected until day 40. Furthermore, it took twice as long (60 days) for the control cultures to reach maximal levels of colony formation compared to the time for HUCB-containing cultures. No CFU-GM were observed in parallel cultures with γ-HUCB cells alone. These results show that HUCB cells can potentiate human hematopoiesis by a mechanism that does not require their own proliferation, and suggest the potential clinical benefits of using HUCB cells therapeutically.

Discussion

HUCB cell therapy rescues mice from irradiation death [18]. It appears that most of the injected HUCB cells initially localize to the liver (Table 2), although the significance for this localization has yet to be determined. The data indicate that HUCB cells mediate the recovery of the endogenous hematopoietic and immunologic systems in NK-depleted, lethally irradiated SJL/J mice. Therefore, this mouse model is a potentially useful experimental system to study a heretofore unrecognized property of HUCB cells in clinical application. Although the HUCB cells injected into lethally irradiated SJL/J mice may initially survive and provide transient protection from acute radiation damage, long-term engraftment of HUCB cells in these mice is unlikely [18,19,24]. Nonetheless, our results using the SJL/J model show that HUCB cells can provide significant benefits for enhanced hematopoietic reconstitution by endogenous stem cells in lethally irradiated mice (Figures 1A, 1B, 3A and 3B). However, there was not a significant effect on hematopoiesis following injection of HUCB cells into mice that received a lower dose (8.0 Gy) of irradiation. There are two possible explanations for these results. First, there may be survival of a sufficient number of stem/progenitor cells in these mice to initiate endogenous hematopoiesis without a need for exogenous stimulation. Secondly, although not tested for, it is possible that HUCB-injected mice can mount an immune response which destroys the HUCB cells before they can fully perform their hematopoiesis-enhancing function. Moreover, these two mechanisms are not mutually exclusive.

Our results also show that HUCB cells exhibit an adjuvant-like activity for reconstitution of selected immune responses (Figures 4, 5, and 6). Especially significant is the enhancement of antigen-specific responses by mice that received HUCB cells (Figure 6). These latter results

are especially important since, in addition to the advantages of HUCB cells over BM cells for transplantation, our results suggest additional clinical benefits of using HUCB cells. The adjuvant-like functions of HUCB cells suggest that they can potentially be used in situations where immune stimulation may be necessary, such as patients with cancer or infectious disease. Furthermore, with regard to application in humans, the immunologic adjuvant property of HUCB gives these cells a dual role, since they can simultaneously engraft and diminish the immunosuppression that can lead to secondary opportunistic infections.

Cell surface markers on HUCB cells may partially explain the combined hematopoietic and immune adjuvant effects observed in this study. Compared to BM, MHC Class II molecules are more densely expressed on HUCB stem cells [31]. Recent studies indicate that MHC Class II is involved in autologous hematopoietic reconstitution in sublethally irradiated dogs [32]. This suggests that part of the hematopoietic effects observed by HUCB cells could be attributed to the high expression of MHC Class II molecules on their stem cells. In addition, CD10 expression in HUCB cells might also be important, since this cell surface marker has an endogenous endopeptidase activity that can utilize as its substrate, several peptides that are relevant to hematopoiesis [33-35].

In vitro, irradiated (γ-) HUCB cells enhance the proliferation of human stem cells (Figure 7). Despite their inability to proliferate, this effect could be mediated by the release of early acting cytokines by the γ-HUCB cells. However, Santois, et al [36] have shown that HUCB cells do not exhibit a dramatic difference in their ability to produce relevant hematopoietic cytokines when compared to peripheral blood mononuclear cells. Therefore, it is more likely that the γ-HUCB cells stimulate the BM stroma to produce cytokines which in turn are capable of

upregulating stem cell activity. The mechanisms by which this stem cell activation occurs is a current focus of our ongoing investigation, since the interaction between HUCB and stromal cells appears important to both the immune adjuvant, as well as the hematopoietic enhancing effects observed in HUCB-injected mice.

In addition to inducing endogenous stem cell proliferation (Figure 7), injection of HUCB cells also leads to reconstitution of differentiated hematopoietic cells as judged by the reappearance of WBC in the peripheral blood of lethally irradiated mice (Figure 1A). This suggests that the presence of HUCB cells not only influences the induction of stem cell proliferation, but also their differentiation. In fact, this is supported by the results of the LTC-IC cultures, where we observed both an accelerated appearance and increased quantity of CFU-GM (Figure 7) in cultures containing γ-HUCB cells. The reason for the decrease at later time periods in the number of CFU-GM in cultures with γ-HUCB is not readily apparent, but could be due to cell death. Trypan blue dye exclusion indicated good viability of cells in these cultures, however, a time-dependent induction of apoptosis cannot be excluded at this time. This area of investigation is currently being addressed, and will provide further insight into the mechanisms of HUCB-mediated endogenous, hematopoietic reconstitution.

The results of this study can be significantly useful for application to the current problems associated with the continued use of allogeneic BM transplantation for treatment of many hematopoietic disorders [37]. Complications associated with GVHD continue to be a medical challenge. Although depletion of T-cells from the donor BM has reduce the incidence of GVHD, loss of this cell population can have negative effects on engraftment, such as an increased rate of reoccurrence of disease [38,39]. This study suggests that HUCB cells could very well be

replacing the engraftment benefits of having T-cells present, but without their deleterious effects of potentiating GVHD. Associated with a lower risk of developing GVHD appears to be a less stringent need for exact donor-recipient HLA matching when HUCB is used for transplantation [40], benefits that have been attributed to the immaturity of HUCB cells [40].

Our results suggest yet another unrecognized benefit of using HUCB cells in lieu of BM for such patients, namely, the ability of HUCB cells to stimulate endogenous hematopoietic repopulation. Because the HUCB cells do not show long-term engraftment in the mouse model, we were able to focus our analysis on this function of HUCB, exclusively. However, in situations where HUCB transplants have been performed in human patients, engraftment of the transplanted cells would actually mask the hematopoiesis-enhancing function of the transplanted HUCB cells. Indeed, since care is usually taken to match the donor-recipient for HLA loci, the hematopoiesis-enhancing function of HUCB would be difficult to measure, and would go largely unnoticed.

In summary, the data presented in this study indicate that HUCB cells facilitate the ability of radioresistant endogenous stem cells to reconstitute the hematopoietic and immunologic systems of lethally irradiated SJL/J mice. Although the mechanisms by which this occurs are yet to be fully determined, the data suggest that HUCB can be used in novel treatment regimens to stimulate endogenous repopulation in patients who currently require BM transplantation. Furthermore, these results suggest that HUCB may also be of potential therapeutic value for immune stimulation. If, as our data suggest, these properties of HUCB cells also occur following transplantation in humans, our observations would be highly significant in lieu of the shortage of human donors, and the widespread controversy regarding xenotransplantation.

Table 1. Phenotypic distribution in cord blood mononuclear cells.

	% of HUCB mononuclear cells (n=7, ±SE)
CD34	2±0.6
CD38	19±5.6
CD34/CD38	3±1.8
CD45	16±5.6
CD3	9±2.8
CD19	6±1.9
CD10	2±0.7
CD14	1.2±0.4
CD56	3.6±0.4

HUCB mononuclear cells were labeled with either FITC- or PE- conjugated monoclonal antibodies. The percentages of labeled cells were determined by FACScan.

Table 2. Distribution of ^{51}Cr-labeled HUCB or BM cells in irradiated SJL/J mice.

Post Injection	% Injected/Organ (cpm) Spleen	Liver	BM	Lung	Blood
HUCB cells					
24 h (n=5)	2.5±0.9	50.0±12.3	0.14±0.04	0.07±0.02	1.0±1.5
48 h (n=6)	2.1±0.9	48.0±10.0	0.13±0.05	0.06±0.03	0.07±0.07
BM cells					
24 h (n=3)	7.2±2.7	15±1.6	0.5±0.2	0.7±0.3	0.6±0.2
48 h (n=5)	4.1±0.6	9.3±1.5	0.2±0.03	0.3±0.1	0.8±0.1

Mice were lethally irradiated 1 day prior to intravenous injection with ^{51}Cr-labeled HUCB cells ($1.5x10^7$ cells = $2.6x10^6$ cpm) or ^{51}Cr-labeled BM cells (10^7 cells = $0.5x10^6$ cpm). Labeling was performed by incubating cells (10^7) with 200 μCi ^{51}Cr in 1 ml volume for 90 min. At 24 and 48 h post-injection, animals were sacrificed and the radioactivity present in various tissues determined.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. The documents should be considered as incorporated by reference in their entirety.

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This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

WHAT IS CLAIMED IS:

1. A method for promoting the growth of the hematopoietic cells of a host comprising administering to said host an effective amount of human umbilical cord blood cells, active fragments thereof, mimics thereof and agonists thereof.

2. The method of Claim 1 wherein said host has previously undergone therapeutic treatment that has caused the myelosuppression of said host, and said method comprises a method for restoring the host hematopoietic system.

3. The method of Claim 1 wherein said human umbilical cord blood cells, active fragments thereof, mimics thereof and agonists thereof, are administered by injection into said host.

4. The method of Claim 1 wherein said human umbilical cord blood cells, active fragments thereof, mimics thereof and agonists thereof are co-cultured *ex vivo* with a quantity of the hematopoietic cells of said host, and said hematopoietic cells so treated are thereafter reintroduced into said host.

5. A method for restoring the immune response of host comprising the method of Claim 1.

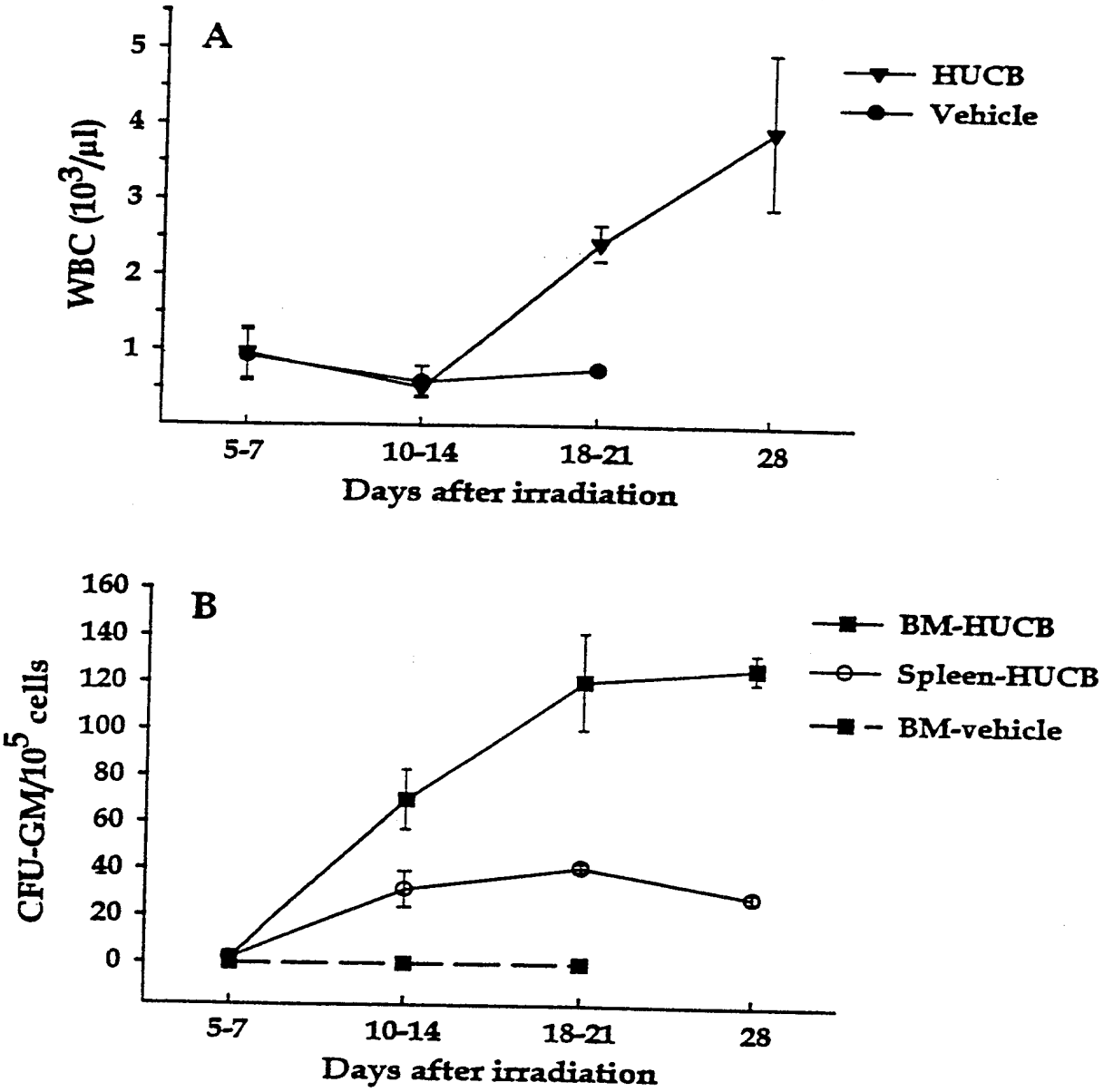
A
WBC (10^3/µl)
5
4
3
2
1
5-7
10-14
18-21
28
Days after irradiation
HUCB
Vehicle
B
CFU-GM/10^5 cells
160
140
120
100
80
60
40
20
0
5-7
10-14
18-21
28
Days after irradiation
BM-HUCB
Spleen-HUCB
BM-vehicle

Figure 1

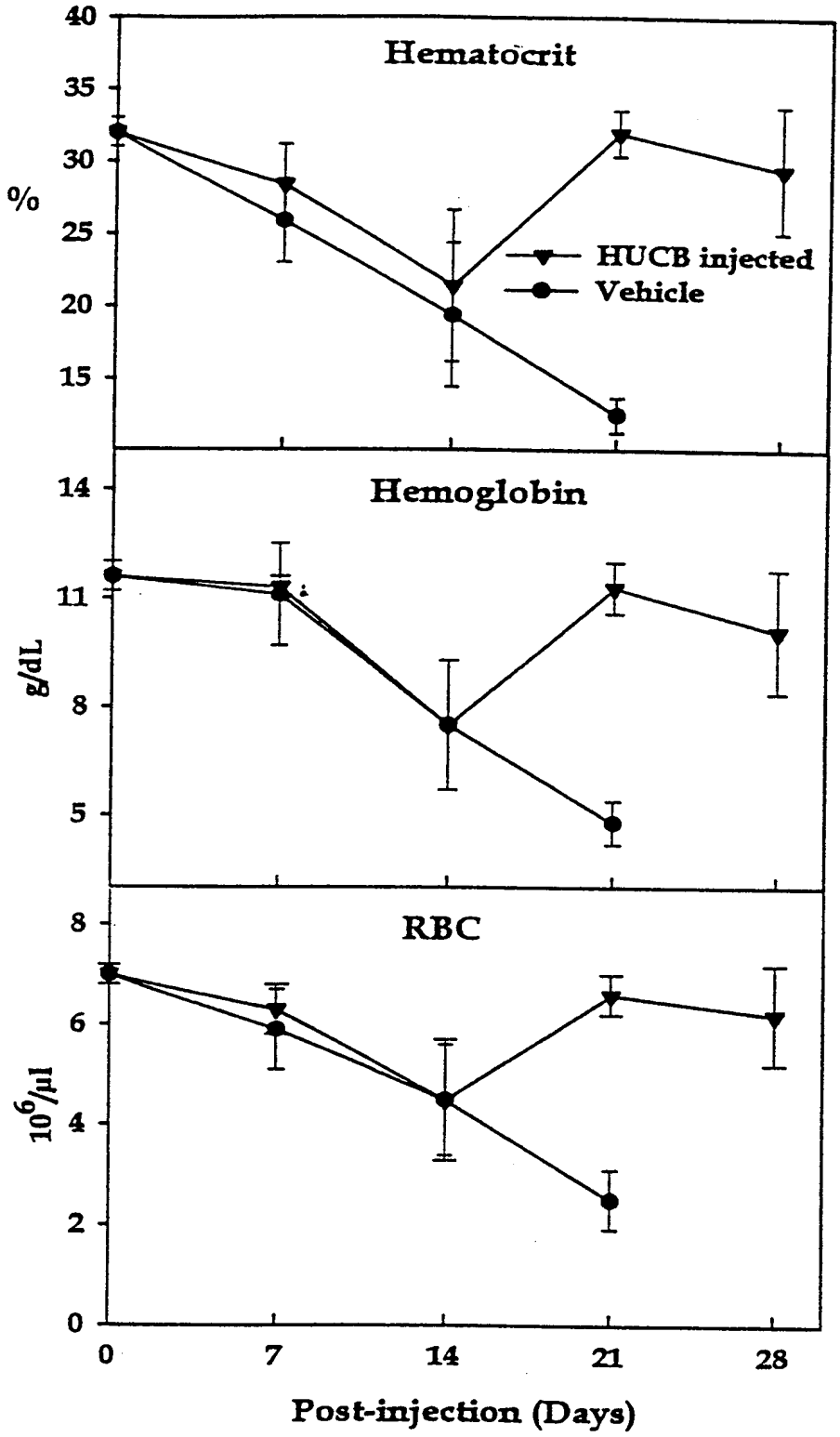
Hematocrit
%
40
35
30
25
20
15
HUCB injected
Vehicle
Hemoglobin
g/dL
14
11
8
5
RBC
$10^6/\mu l$
8
6
4
2
0
0
7
14
21
28
Post-injection (Days)

Figure 2

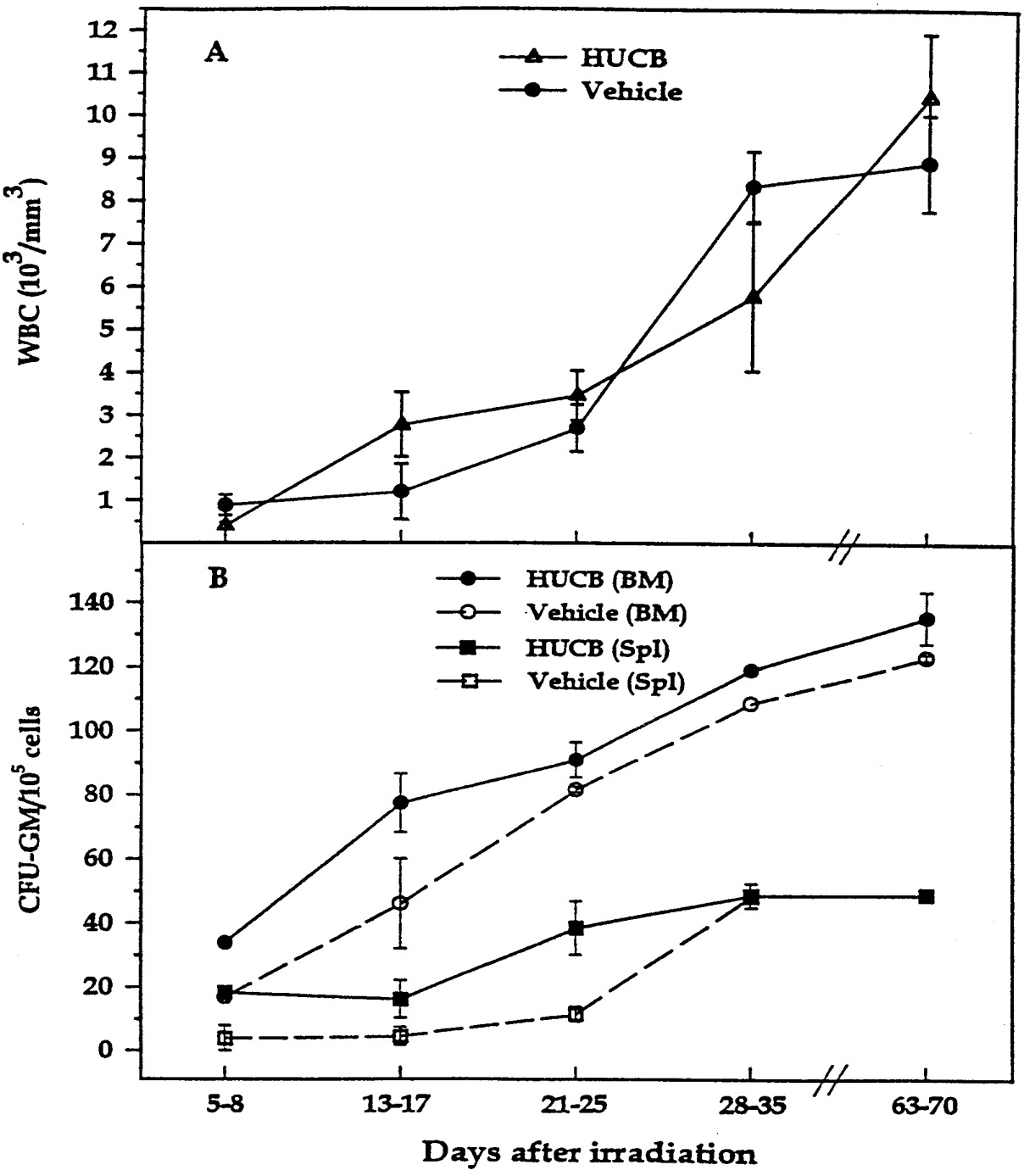
A
HUCB
Vehicle
WBC (10^3/mm^3)
12
11
10
9
8
7
6
5
4
3
2
1
B
HUCB (BM)
Vehicle (BM)
HUCB (Spl)
Vehicle (Spl)
CFU-GM/10^5 cells
140
120
100
80
60
40
20
0
5-8
13-17
21-25
28-35
63-70
Days after irradiation

Figure 3

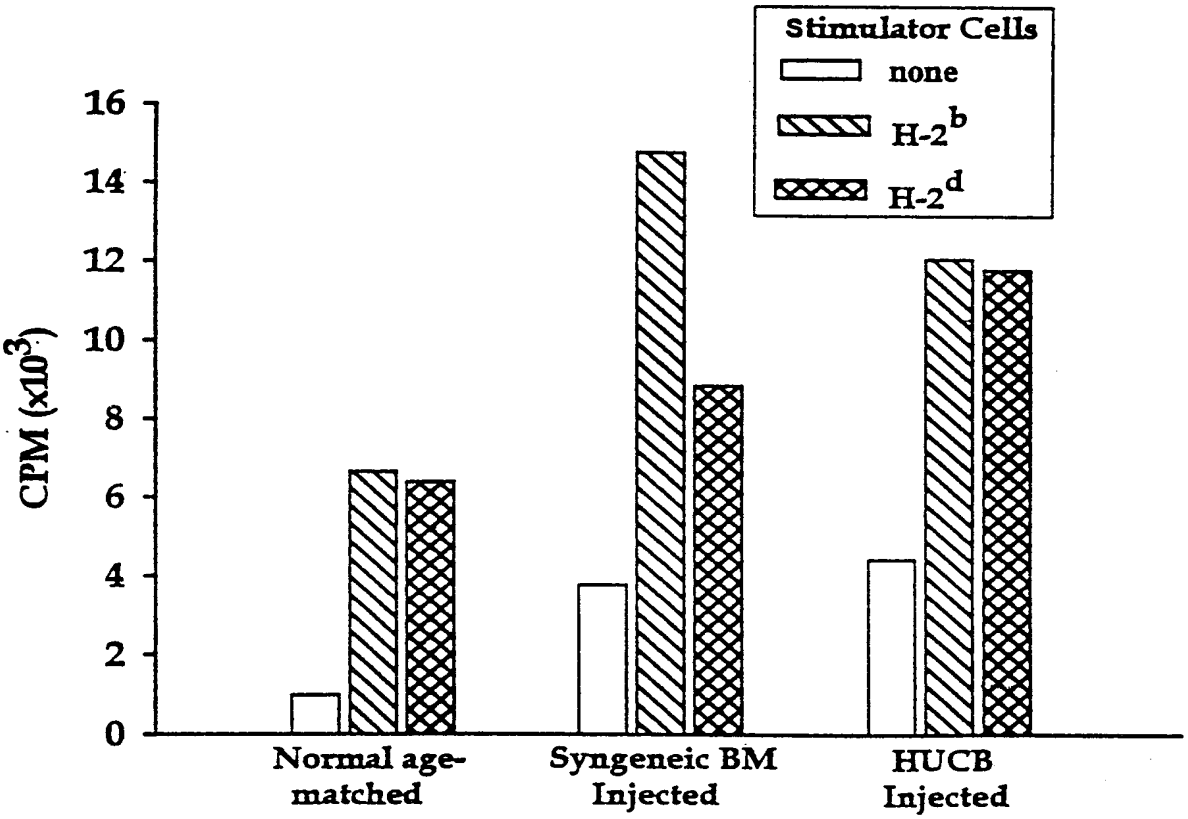
Stimulator Cells
none
H-2b
H-2d
CPM (x10³)
16
14
12
10
8
6
4
2
0
Normal age-matched
Syngeneic BM Injected
HUCB Injected

Figure 4

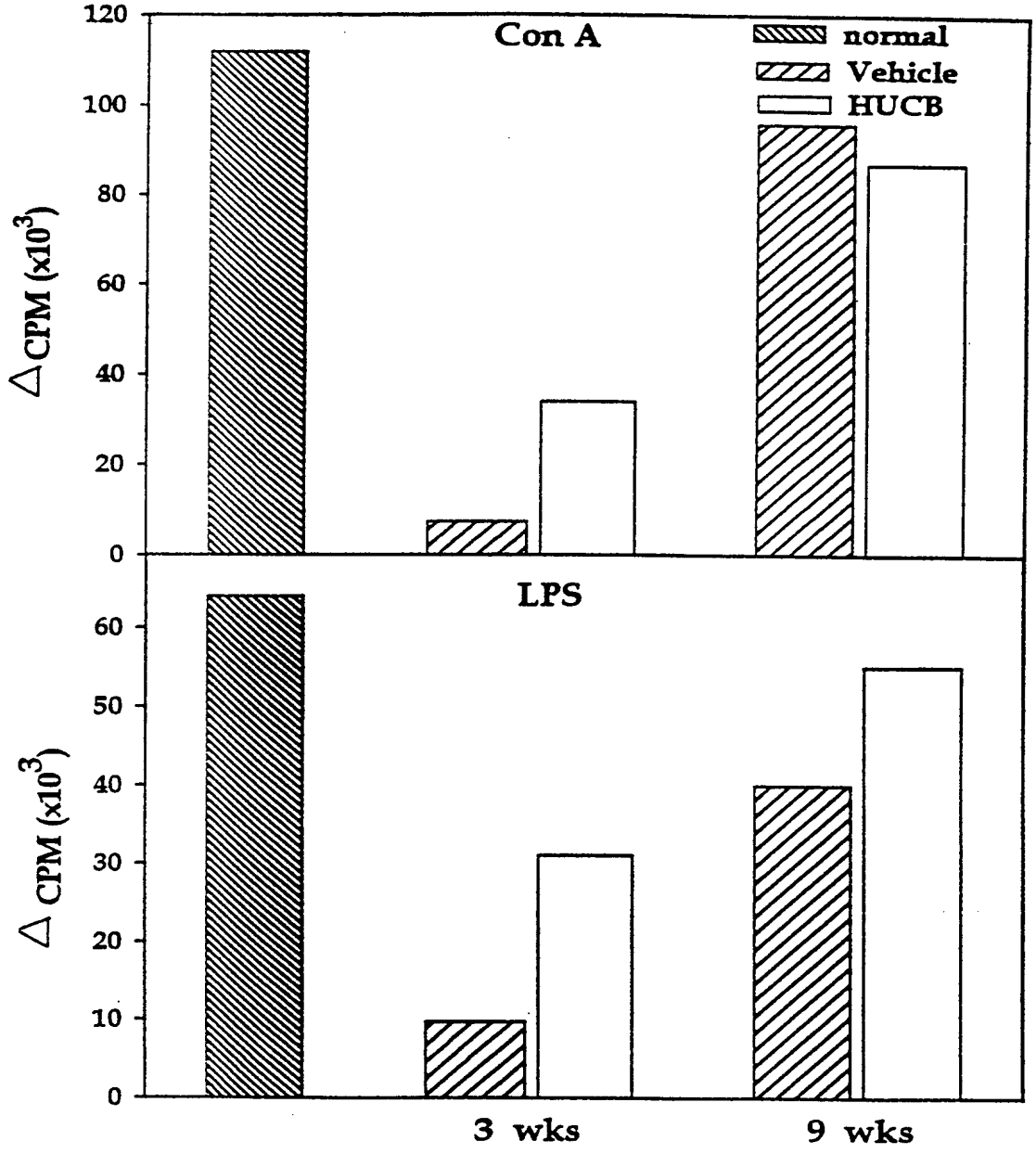
Con A
normal
Vehicle
HUCB
Δ CPM (x10³)
120
100
80
60
40
20
0
LPS
Δ CPM (x10³)
60
50
40
30
20
10
0
3 wks
9 wks

Figure 5

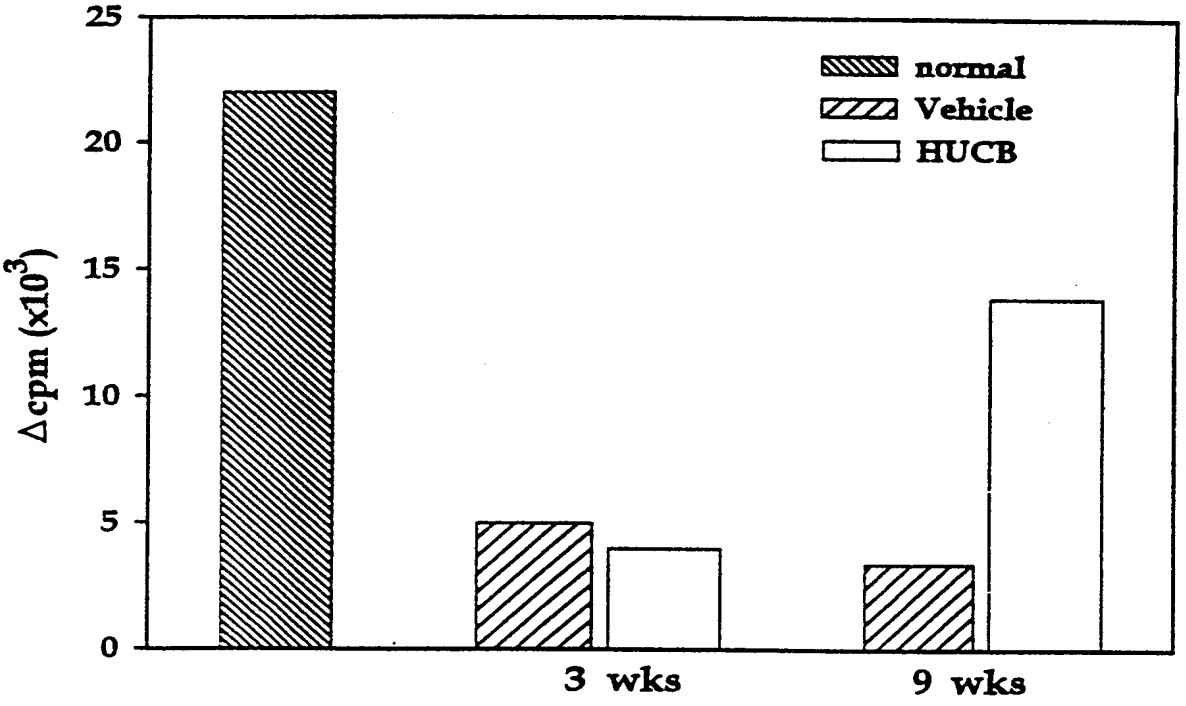

Figure 6

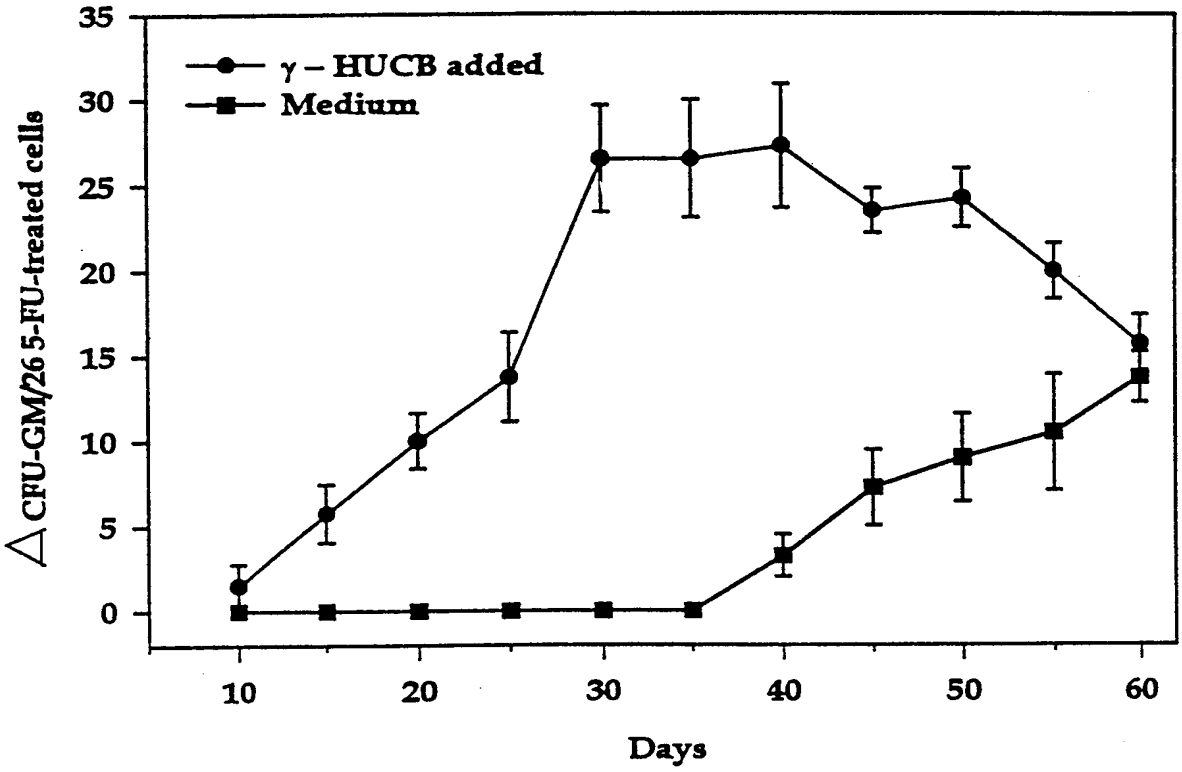
γ – HUCB added
Medium
△ CFU-GM/26 5-FU-treated cells
Days
0
5
10
15
20
25
30
35
10
20
30
40
50
60

Figure 7

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 98/25697

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/44 A61K35/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAGNER J E: "Umbilical cord blood transplantation: overview of the clinical experience 'see comments!." BLOOD CELLS, (1994) 20 (2-3) 227-33;DISCUSSION 233-4, XP002101086 see the whole document ---	1-5
X	DOVAT S ET AL: "The use of umbilical cord blood stem cells for hematopoietic reconstitution." WESTERN JOURNAL OF MEDICINE, (1997 MAY) 166 (5) 342-3, XP002101087 see the whole document ---	1-5

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 April 1999	21/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 98/25697

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KATZENSTEIN, H. M. ET AL: "Haploidentical related umbilical cord blood stem cell transplant in a child with acute non-lymphocytic leukemia." BONE MARROW TRANSPLANTATION, (1997) VOL. 19, NO. 8, PP. 765-769, XP002101088 see the whole document ---	1-5
X	WO 91 16062 A (ENDE N.) 31 October 1991 see claims 1,13 ---	1-5
P,X	LAPORTE J P ET AL: "Unrelated mismatched cord blood transplantation in patients with hematological malignancies: a single institution experience." BONE MARROW TRANSPLANTATION, (1998 JUL) 22 SUPPL 1 S76-7, XP002101089 see the whole document -----	1-5

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INTERNATIONAL SEARCH REPORT

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-5
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-5 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Interr nal Application No
PCT/US 98/25697

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9116062 A	31-10-1991	AU 7780791 A	11-11-1991